

AN OPTIMIZED LYMPHOCYTE BLASTOGENESIS ASSAY FOR DETECTING THE RESPONSE OF CONTACT SENSITIZED OR PHOTOSENSITIZED LYMPHOCYTES TO HAPTEN OR PHOTOHAPTEN MODIFIED ANTIGEN PRESENTING CELLS

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Abstract—*In vitro* assays for predictive identification of contact sensitizers of photosensitizers are more quantifiable than current skin test methods and have the potential to reduce animal use. Mice were sensitized to various strong contact allergens, then an optimized lymphocyte blastogenesis assay was used for detecting the proliferation of lymph node lymphocytes from those mice in response to soluble antigen or hapten-modified antigen presenting cells in culture. *In vivo* sensitization to oxazolone (OXAZ), a prototype sensitizer, resulted in poor *in vitro* lymphocyte blastogenesis to a solubilized OXAZ preparation. To increase the assay sensitivity, dendritic cells from the draining lymph nodes of OXAZ-painted mice or OXAZ-modified Langerhans cell-enriched cultured epidermal cells (EC) were used as antigen presenting cells. This increased the OXAZ-specific response greater than 10-fold and 50-fold, respectively. This approach has since been used to demonstrate significant, albeit smaller, responses to weaker contact allergens such as nickel sulphate. EC were also used as antigen-presenting cells for *in vitro* lymphocyte blastogenesis assays designed to detect photoallergens. Lymphocytes from mice photosensitized to tetrachlorosalicylanilide (TCSA) showed a significantly increased response to EC previously photohaptened by treatment with TCSA plus ultraviolet A (UVA), as compared with lymphocytes cultured with EC treated with TCSA but no UVA. The lymphocyte blastogenesis assay may have potential as a predictive screen to identify contact and photocontact allergens.

Introduction

Standard testing methods for predictive assessment of contact sensitization (CS) and contact photosensitization (CPS) are limited to various guinea-pig testing methods that measure cutaneous erythema and/or oedema in suitably induced and challenged animals (Andersen and Maibach, 1985; Gerberick and Ryan, 1989; Maurer, 1984). The limitations of these methods are that they rely on subjective skin reaction grades thereby producing limited quantitative data. In addition, these methods do not easily distinguish weak sensitization from irritation reactions and they are time consuming and require relatively large numbers of animals. The use of the mouse ear-swelling assay has recently been proposed as a more quantifiable method for predictive testing, but has provided somewhat varied results (Gad *et al.*, 1986; Maisey and Miller, 1986; Moller, 1984).

Thus, we have evaluated the use of an immunologically-based, *in vitro* lymphocyte proliferation (blastogenesis) assay for identification of contact allergens and photoallergens. Already used extensively for mechanistic studies of the cellular immune response, these assays have provided some utility in

clinical diagnosis of contact allergy (Res *et al.*, 1987). Preliminary data have also indicated their potential use in the identification of contact allergens (Kimber *et al.*, 1986; Robinson, 1989). We report here on the development of the *in vitro* lymphocyte blastogenesis assay as a method for confirming sensitization in contact allergen or photoallergen treated mice.

Materials and Methods

Mice. Female BALB/c mice, 2–4 months old were used. Three to five animals were used per test group and all *in vitro* assays were conducted with cells from individual mice unless otherwise indicated.

Test antigens. Contact allergens studied included 1-chloro-2,4-dinitrobenzene (DNCB), 1-fluoro-2,4-dinitrobenzene (DNFB), 1-chloro-2,4,6 trinitrobenzene (TNB), 4-ethoxymethylene-2-phenyl-oxazol-5-one (oxazolone (OXAZ)) and nickel sulphate (NiSO₄). For *in vitro* studies DNCB, DNFB and TNB were replaced by 2,4-dinitrobenzene sulphonic acid (DNBS) and 2,4,6-trinitrobenzene sulphonic acid (TNBS). Contact photoallergens studied included 3,3',4',5-tetrachlorosalicylanilide (TCSA) and 6-methylcoumarin (6-MC).

In vivo sensitization and ear-swelling assay. Sensitization was induced by making one or more daily applications of 25–50 µl of the test material to the shaved dorsal or ventral surface of the mouse. Control mice were either left untreated or were treated in the same fashion with vehicle alone. On days 4–5 after induction, test and control animals were

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Abbreviations: DNBS = 2,4-dinitrobenzene sulphonic acid; DNCB = 1-chloro-2,4-dinitrobenzene; DNFB = 1-fluoro-2,4-dinitrobenzene; EC = epidermal cells; OXAZ = oxazolone; TCSA = tetrachlorosalicylanilide; TNBS = 2,4,6-trinitrobenzene sulphonic acid; TNB = 1-chloro-2,4,6 trinitrobenzene; UV = ultraviolet.

challenged by applying 5 μ l of test antigen solution on each side of one ear and 5 μ l of vehicle on each side of the other ear. Ear thickness measurements were taken for each ear using a caliper just prior to challenge. Measurements were repeated 24 hr (and sometimes 48 hr) after challenge. Ear swelling was calculated by subtracting the pre-challenge value from the post-challenge value and then further subtracting any swelling recorded for the vehicle-challenged ear (never more than 10% above pre-challenge value) from the swelling recorded for the antigen-challenged ear.

In vivo photosensitization. For photosensitization, we developed a mouse ear-swelling model for photoallergy testing that is a modification of previously reported models (Granstein *et al.*, 1983; Maguire and Kaidbey, 1982; Takigawa and Miyachi, 1982). *In vivo* test materials were prepared as w/v% solutions in acetone for both induction and challenge. Balb/c mice were treated with 200 mg cyclophosphamide/kg body weight by ip injection three days prior to the first induction and then induced by applying 50 μ l of the test material in vehicle to the shaved dorsal surface on days 0, 1 and 2. Thirty min following application of the test material, the mice were exposed to 10 J/cm² ultraviolet A (UVA) radiation supplied by a bank of Sylvania F40/350BL fluorescent blacklights and 45 mJ/cm² UVB radiation supplied by eight Philips F40UVB fluorescent sunlamps. Seven days after the first induction, the mice were

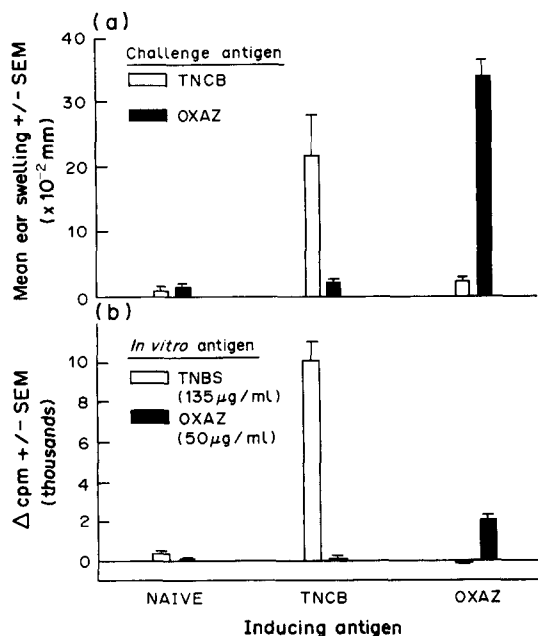


Fig. 1. Three mice per group received single induction applications of 1% TNCB or 2% OXAZ in 4:1 acetone:olive oil (A:OO) or 4:1 A:OO alone (vehicle control). Four days later they were challenged on one ear with 0.2% TNCB or 0.5% OXAZ and ear-thickness increases were recorded 24 hr later (a). Lymphocyte suspensions were then prepared from the draining lymph nodes of individual animals and cultured at 4×10^5 cells/culture with the indicated concentrations of TNBS or OXAZ. The cultures were incubated for 3 days, labelled with [³H]thymidine, and [³H] incorporation was determined after an additional 24-hr incubation (b).

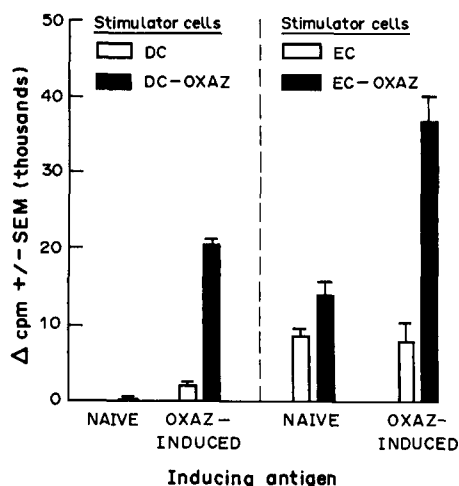


Fig. 2. In two separate experiments, three to four mice per group received single induction applications of 2% OXAZ in 4:1 A:OO (naive animals served as controls). Five days later the mice were killed and lymphocyte suspensions were then prepared from the draining lymph nodes of individual animals and cultured at 4×10^5 cells/culture with dendritic lymph node cells from naive (DC) or OXAZ painted mice (DC-OXAZ) or with Langerhans cell enriched cultured EC or EC modified by *in vitro* incubation with OXAZ (EC-OXAZ) (see Materials and Methods). The cultures were incubated for 3 days, labelled with [³H]thymidine, and [³H] incorporation was determined after an additional 24-hr incubation.

challenged by applying 8 μ l of the test material in vehicle to each side of both ears. Thirty minutes after test material application, the mice were irradiated as described above.

Preparation of antigen-presenting cells. In some experiments, dendritic lymph node cells from naive or antigen-treated mice were used as antigen-presenting cells as described (Robinson, 1989). Langerhans cells enriched cultured epidermal cells (EC) were obtained from ear and truncal skin of normal mice as described (Robinson, 1989). For antigen (hapten) modification of antigen-presenting cells, portions of the EC were suspended in Hank's balanced salt solution containing either 5 mM-TNBS, 2 mM-solubilized OXAZ (see Robinson, 1989, for solubilization procedure), or 5 mM-NiSO₄ and incubated (37°C, 5% CO₂) for 30 min. For photohapten modification, EC were irradiated, in the presence of the photoallergen, with 2 J/cm² UVA using a 1000 W solar simulator (Oriol Corp., Stratford, CT, USA). TCSA was solubilized in absolute ethanol and then diluted to the appropriate concentration (0.05 mM) in a balanced salt solution. Stimulator cells included EC (without treatment), EC first irradiated with UVA and then treated with TCSA, and EC treated with TCSA and UVA simultaneously. All such antigen treated cells were then washed, counted, and resuspended in culture medium for addition to assay cultures.

Blastogenesis assay cultures. Mice were killed after recording the 24-hr (and sometimes 48-hr) and post-challenge ear-thickness measurements. Peripheral lymph nodes (axillary, brachial and inguinal) were pooled from individual mice or from all mice within a given test or control group and cell suspensions

prepared. Assay cultures were prepared and blastogenesis assessed as described (Robinson, 1989) using [^{3} H]thymidine uptake to quantify lymphocyte blastogenesis.

Results

Optimization of lymphocyte blastogenesis assay for contact sensitizers

Our initial studies with strong contact allergens showed a clear discrepancy between *in vivo* sensitization test (ear-swelling) results and the follow-up blastogenesis assay results using lymphocytes obtained from those animals (Fig. 1). The ear-swelling data showed strong and antigen-specific responses to both TNCB and OXAZ (Fig. 1a). However, the *in vitro* responses to TNBS and solubilized OXAZ were quite different. Again, the responses were antigen-specific; however, the solubilized OXAZ triggered a relatively poor response in lymphocytes from OXAZ-sensitized mice despite the strong *in vivo* response (Fig. 1b).

To improve the assay sensitivity for OXAZ, we evaluated various options for cellular presentation of the OXAZ antigen to responder lymphocytes. Initial efforts using adherent spleen cells gave only a 2–3-fold increase in activity (data not shown). However, utilizing dendritic cells from the draining lymph nodes of OXAZ-painted mice (Kinnaid *et al.*, 1989; Macatonia *et al.*, 1986), or OXAZ-modified Langerhans cell enriched cultured EC (Robinson, 1989; Shimada *et al.*, 1987), increased the response 10–20-fold relative to solubilized OXAZ alone (Fig. 2). Further optimization efforts (e.g. use of nylon wool non-adherent lymphocytes) increased the CPM response by >50-fold (Robinson, 1989).

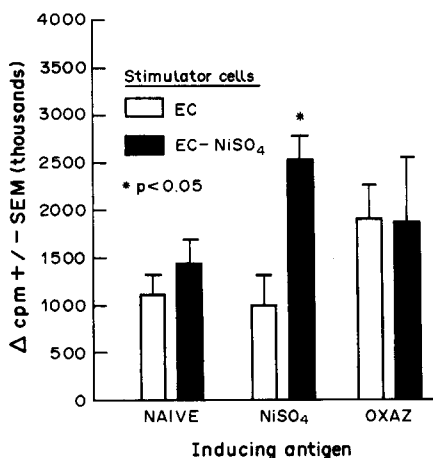


Fig. 3. Five mice received five twice-daily inductions of 20% NiSO₄ in 30% aqueous ethanol (naïve animals served as controls). Five additional mice received one induction application of 2% OXAZ in 100% ethanol. Four days after the last NiSO₄ application or single OXAZ treatment, lymphocyte suspensions were prepared from the draining lymph nodes of individual animals and cultured at 4×10^5 cells/culture with the indicated antigen presenting cells (see Materials and Methods). The cultures were incubated for 2 days, labelled with [^{3} H]thymidine, and [^{3} H] incorporation was determined after an additional 24-hr incubation.

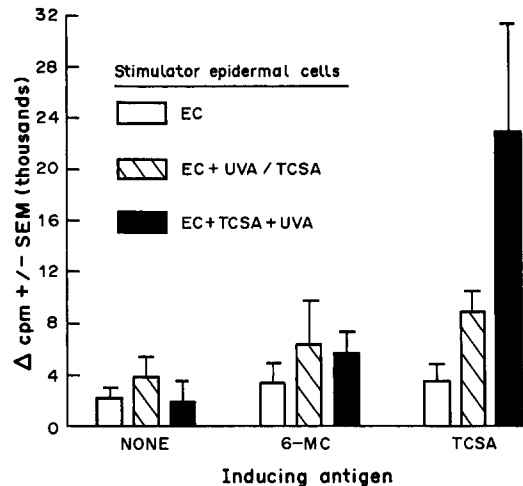


Fig. 4. Four mice per group were photosensitized to TCSA or 6-MC. Mice received three induction treatments of either 1% TCSA in acetone plus 10 J/cm² UVA and 45 mJ/cm² UVB or 20% 6-MC in acetone plus 10 J/cm² UVA and 45 mJ/cm² UVB. Seven days after the first induction, mice were challenged with either 1% TCSA in acetone plus UVA/UVB or 10% 6-MC in acetone plus UVA/UVB. At 24 hr post-challenge, the mice were killed along with four naïve controls, and lymphocyte suspensions were prepared from the draining lymph nodes of individual animals and cultured at 3×10^5 cells/culture with the indicated antigen-presenting cells (see Materials and Methods). The cultures were incubated for 2 days, labelled with [^{3} H]thymidine, and [^{3} H] incorporation was determined after an additional 24-hr incubation. Each point represents the mean value of four animals.

Nickel sulphate (NiSO₄) was also evaluated in this model to determine the potential of this assay to identify a weaker contact sensitizer. In preliminary studies (Fig. 3), lymphocytes from mice subjected to 10 induction applications of NiSO₄ (twice a day for 5 days) showed a small but significant response to NiSO₄-modified EC relative to the autologous response with unmodified EC. This preferential response to NiSO₄-modified EC was not observed in lymphocytes from naïve mice or mice sensitized to the unrelated antigen, OXAZ.

Lymphocyte blastogenesis assay for contact photosensitizers

We have also examined photoallergy *in vitro* using TCSA photohapten-modified EC and lymphocytes from TCSA photosensitized mice. We observed a significant increase in the blastogenesis response of TCSA sensitized lymphocytes when cultured with epidermal cells that were photohapten-modified with TCSA and UVA radiation as compared with the responses to either epidermal cells irradiated with UVA radiation and then treated with TCSA or epidermal cells receiving no treatment (Fig. 4). In addition, lymphocytes from untreated mice or mice sensitized with 6-MC demonstrated a significantly lower response to EC photohapten-modified with TCSA and UVA, which indicated the specificity of the reaction.

Discussion

The purpose of these studies was to develop an *in vitro* lymphocyte blastogenesis assay for confirming or better quantifying induced *in vivo* sensitization to contact allergens and photoallergens. Though ear-swelling responses were consistently detected for the strong contact sensitizer OXAZ, lymphocytes from OXAZ-sensitized mice responded poorly to solubilized OXAZ *in vitro*. The blastogenesis response to OXAZ was significantly increased using OXAZ-modified epidermal cells to present the relevant hapten to the responder lymphocytes. A strong blastogenesis response was also observed using dendritic lymph node stimulator cells from OXAZ-induced mice. This latter approach may prove useful when testing water insoluble chemicals or chemicals directly toxic to epidermal cells *in vitro*.

The *in vitro* methodology was also able to detect the weaker contact allergen, nickel sulphate, as well as the contact photoallergen TCSEA. Nickel sulphate is widely used to elicit skin reactions in persons sensitized to metallic nickel (Marzulli and Maibach, 1987); however, as an inducing material it is classified as only a weak to moderate sensitizer (Kligman, 1966; Magnusson and Kligman, 1969; Zissu *et al.*, 1987). Using repeated induction exposures to NiSO₄, we were able to induce sensitization as demonstrated by a significant and antigen-specific blastogenesis response to NiSO₄-modified EC. In addition, using an appropriate *in vitro* irradiation technique, we have been able to photohapten-modify epidermal antigen-presenting cells and demonstrate (for the first time) a photoallergen-specific blastogenesis response by lymphocytes from photoallergen sensitized mice.

These data indicate that the antigen stimulated lymphocyte blastogenesis response assay has the potential to aid in identifying potential human contact allergens and photoallergens. This method may prove useful for investigating the skin sensitization potential of chemicals relevant to consumer and occupational exposure and reduce the number of laboratory animals required for skin sensitization safety assessments.

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